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Specific Immune Serum to the Epstein-Barr Virus DNA Polymerase

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Epstein-Barr virus (EBV) DNA polymerase was released from phorbol ester-treated tamarin (*Saguinus oedipus*) cells (B95-8) and prepared for use as an antigen by sequential column chromatography with DEAE–Sephadex A-25, DEAE-cellulose, phosphocellulose, and single-stranded DNA cellulose. Proteins from single-stranded DNA cellulose with DNA polymerase activity in 100 mM ammonium sulfate were mixed with complete Freund adjuvant and injected intradermally into rats and rabbits. Immune sera that were screened for specific antibody by indirect immunofluorescence procedures reacted with approximately 3% of the cells in EBV-producer cultures (B95-8 and P3HR-1) but not with EBV genome-negative cells (BJAB). In functional enzyme assays, immune sera or the immunoglobulin fraction inhibited the activity of purified EBV DNA polymerase 90%. Inhibition of enzyme activity was not affected by absorption of immune sera with insoluble matrices of proteins prepared with tamarin and human cells which lacked the EBV genome. Cellular DNA polymerase alpha was not inhibited by immune sera to the EBV enzyme.

Several enzyme activities that are associated with nucleic acid metabolism are induced during Epstein-Barr virus (EBV) replication. Among these are a DNA polymerase (2, 7, 9, 13, 20, 23), a DNase (5, 6, 22, 32), a ribonucleotide reductase (11), a thymidine kinase (4, 8, 18, 21, 26), and a dUTPase (33). There are no reports of specific immune sera to any of these enzymes. Immune sera would be valuable in studies concerning the role that each enzyme plays in the scheme of virus replication.

The purpose of this study was to obtain antiserum to the EBV DNA polymerase. The EBV enzyme differs from cellular DNA polymerases in chromatographic properties and sensitivity to DNA polymerase inhibitors. The immediate product of its synthesis is covalently closed circular DNA (28, 29). This circular DNA can be distinguished from the circular DNA of latently infected cells (15), which is complexed with histones (28). The circular and integrated forms (1, 17) of latent viral DNA are presumably synthesized by host DNA polymerases, whereas the circular DNA that is synthesized by the viral enzyme (29) is likely to be a precursor that is cleaved to yield the linear DNA found in virions (25).

To prepare antiserum to the EBV DNA polymerase, we diluted B95-8 cells (19) in exponential growth to 5×10^5 cells per ml with RPMI 1640 medium supplemented with 5% fetal bovine serum. Cells were exposed to the tumor promoter and EBV-inducing agent phorbol 12-myristate 13-acetate (34) at 20 ng/ml. After 48 h at 37°C, 5×10^8 cells were washed once with phosphate-buffered saline (130 mM sodium chloride, 50 mM potassium chloride, 10 mM sodium phosphate [pH 7.4]), suspended in 4.5 ml of buffer A (50 mM Tris hydrochloride [pH 8.0], 10 mM magnesium chloride, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2% Nonidet P-40, 20% glycerol, 300 mM ammonium sulfate), and sonicated at 0°C for 2 min. The sonicate was clarified at 25,000 × *g* for 15 min at 4°C. The supernatant was enriched for the EBV enzyme by column chromatography methods used to purify EBV (7) and herpes simplex virus type 1 (24) DNA polymerases. Sequential column chromatography of a clarified B95-8 cell lysate is shown in Fig. 1. Column fractions were assayed in the presence of 100 mM ammonium sulfate to detect viral DNA polymerase activity (7).

DEAE–Sephadex A-25 and DEAE-cellulose separate cellular DNA polymerases beta and alpha, respectively, from the EBV DNA polymerase (7). Viral DNA polymerase activity in fractions 15 to 21 from DEAE–Sephadex A-25 (Fig. 1A) were pooled, made 500 μg/ml with bovine serum albumin, and applied to a column of DEAE–cellulose. Fractions 9 to 16 from DEAE–cellulose (Fig. 1B) were chromatographed on P-11 phosphocellulose. Fractions 22 to 27 from phosphocellulose (Fig. 1C) were applied to a column of single-stranded DNA cellulose. Fractions 26 to 29 from DNA cellulose (Fig. 1D) were pooled, frozen at −70°C, and subsequently used for immunizations. The concentration of protein was determined to be 54 μg/ml by the procedure of Lowry et al. (16). DNA polymerase activity was not detected above the background level when a clarified lysate of EBV genome-negative BJAB cells (14) was analyzed by these methods. This observation agrees with the published findings of other investigators (13).

Three-month-old Norway Brown rats and juvenile (3.18-kg) New Zealand White rabbits were immunized by repeated intradermal injections on the hindquarter (rat) or back (rabbit). Each inoculum consisted of 0.25 ml of complete Freund adjuvant mixed with 0.25 ml of the antigen preparation (5 μg) diluted with phosphate-buffered saline. Animals were boosted once a month and bled from the tail (rat) or ear (rabbit) 1 week after each booster. Preimmune sera were collected from each animal 1 week before immunizations were begun. Antibody to the EBV DNA polymerase was detected 4 weeks after the first inoculation. Maximum antibody titers varied among the animals, but they were usually achieved after two injections of the antigen.

Sera from immune animals were screened for antibody to the viral DNA polymerase by a functional assay for the enzyme in vitro under standard conditions (Fig. 1). Each assay used 5 to 35 μl of whole serum or the immunoglobulin fraction, which was obtained by ammonium sulfate precipitation. Antibody preparations were dialyzed before use against 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesul-

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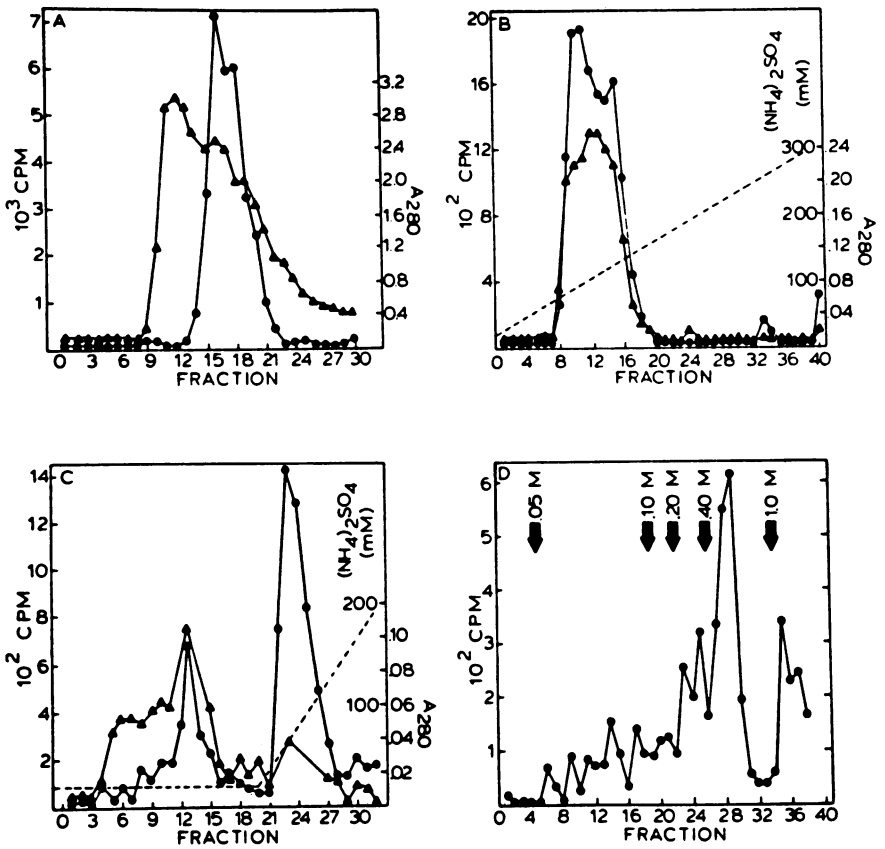

FIG. 1. Preparation of enzyme antigen. Soluble proteins from virus-induced B95-8 cells were enriched for the EBV DNA polymerase by sequential column chromatography with DEAE–Sephadex A-25 (A), DEAE-cellulose (B), P-11 phosphocellulose (C), and single-stranded DNA cellulose (D). Column fractions were assayed for viral enzyme activity under standard conditions: 100-μl reaction mixtures contained 100 mM ammonium sulfate, 7 mM Tris hydrochloride (pH 8.0), 4 mM magnesium chloride, 100 μM each dATP, dCTP, and dGTP, 10 uM [^{3}H]dTTP (10^3 cpm/pmol), 2% glycerol, 70 μg of bovine serum albumin dialyzed against water), 13 μg of activated, native salmon sperm DNA (27), and 5 to 25 μl of enzyme. Reaction mixtures were incubated for 30 min at 37°C, and the reactions were stopped with an equal volume of ice-cold 20% trichloroacetic acid containing 20 mM sodium PP_i. When serum proteins were incubated with the enzyme (See Fig. 2), an equal volume of stop buffer (1% Sarkosyl, 500 μg of native salmon sperm DNA per ml, 20 mM disodium EDTA [pH 7.0], 200 μg of proteinase K per ml) was added to each reaction mixture, and incubation at 37°C was continued for an additional 30 min before precipitation with trichloroacetic acid. Symbols: ●, incorporation of [^{3}H]dTMP; ▲, A_{280}. Arrows in panel D indicate changes in the concentration of potassium chloride.

fonic acid (HEPES) buffer (pH 8.0), containing 50 mM sodium chloride.

The enzyme that was used to screen sera for antibody was obtained from an exponentially growing B95-8 culture that was diluted to 5×10^5 cells per ml with RPMI 1640 medium supplemented with 5% fetal bovine serum and exposed to 20 ng of phorbol 12-myristate 13-acetate per ml and 2 mM *n*-butyric acid (31). Maximum expression of the enzyme was detected 48 h after exposure to the inducers. Cells were washed twice with phosphate-buffered saline at this time and frozen at −70°C.

The enzyme was released from a pellet of 2×10^{10} cells, purified by double-stranded DNA cellulose and phosphocellulose column chromatography, and then stored in 50% glycerol at −20°C as described previously (13). The B95-8 viral enzyme recovered by these procedures was inhibited by phosphonoacetic acid and stimulated by ammonium sulfate and potassium chloride.

The effect of immune serum on the activity of the B95-8 viral enzyme is shown in Fig. 2. Each assay contained 2.3 mg of rat serum protein. Only the quantities of immune serum (0.5 to 2.3 mg) are shown. Preimmune serum was added to reactions when appropriate to provide 2.3 mg of total serum protein for each assay. Each assay was performed with the same quantity of serum protein because preliminary findings revealed that there was a differential protection of enzyme activity when the quantities of protein (nonimmune serum or bovine serum albumin) in each reaction were not the same.

The data in Fig. 2 showed that the inhibition of viral enzyme activity was related to the concentration of immune serum in the reaction. Enzyme and serum were not preincubated. The value at 100% activity (0 mg of immune serum protein) represents the incorporation of dTMP when 2.3 mg of preimmune serum was incubated with the enzyme. In the presence of 2.3 mg of immune serum, enzyme activity was inhibited 90%. Ninety percent inhibition of enzyme activity was also achieved with the immunoglobulin fraction of rat serum and when immune rabbit serum was incubated with the enzyme.

Immune sera were screened for antibody by indirect immunofluorescence procedures as described previously (10). In the EBV-producer cultures B95-8 and P3HR-1 (12), approximately 3% of the cells exhibited intense fluorescence. Whole-cell fluorescence predominated, but some of

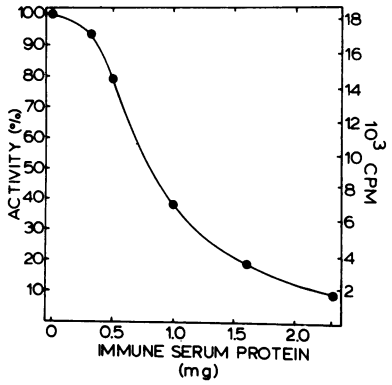

FIG. 2. Inhibition of EBV DNA polymerase activity by immune serum. The viral enzyme from B95-8 cells was used to screen sera for specific antibody to the EBV DNA polymerase. The enzyme was released from virus-induced cells and purified with double-stranded DNA cellulose and phosphocellulose as described in the text. In each assay the quantity of serum protein was identical (2.3 mg). Only the quantities of immune serum are shown. Preimmune serum was added to reactions when appropriate to provide 2.3 mg of total protein for each assay. The data corresponding to 0 and 2.3 mg on the abscissa represent the quantities of dTMP incorporated when the viral enzyme was incubated with 2.3 mg of preimmune serum and 2.3 mg of immune serum, respectively.

the cells exhibited only nuclear fluorescence. After exposure to phorbol 12-myristate 13-acetate and *n*-butyrate (31), the number of fluorescent cells in EBV-producer cultures increased. Cells in EBV-producer cultures exposed to preimmune serum exhibited background fluorescence only. Furthermore, only background fluorescence was observed when sera were screened with EBV genome-negative BJAB cells. BJAB cells exhibited a slightly higher general background fluorescence with immune serum than with preimmune serum. This observation may reflect the carry-over of cellular protein or bovine serum albumin during column chromatography of the enzyme antigen.

Preimmune and immune rabbit sera were absorbed with insoluble matrices of proteins obtained from human cells (BJAB) and tamarin (*Saguinus oedipus*) peripheral blood cells. The protein matrices were covalently cross-linked with ethylchloroformate as described previously (3). When nonabsorbed and absorbed sera were compared, they inhibited viral DNA polymerase activity 93 and 97% respectively. Matrices that were prepared with proteins from virus-induced B95-8 cells reduced, by approximately 10%, the enzyme-inhibiting activity of immune sera. Thus, absorption with proteins from EBV genome-negative human and tamarin cells did not remove the antibodies from immune serum which inhibited viral DNA polymerase activity. These findings indicate that the antigens are expressed only by EBV-transformed cells.

TABLE 1. Effect of immune rabbit serum on the EBV DNA polymerase and cellular DNA polymerase alpha[a]

Enzyme	Enzyme activity (cpm)[b]		% Activity[c] remaining
	Preimmune[d]	Immune[e]	
EBV DNA polymerase	5,559	2,993	54
Cellular DNA polymerase alpha	7,048	7,538	107

[a] See text for purification of cellular DNA polymerase alpha.
[b] [^{3}H]dTMP incorporated; mean values for triplicate assays.
[c] (Preimmune value/immune value) × 100.
[d] The enzyme was incubated with 1.5 mg of preimmune serum protein per assay.
[e] The enzyme was incubated with 1.5 mg of immune serum protein per assay; immune serum was obtained from animals immunized with the antigen described in Fig. 1.

The effects of preimmune and immune sera on the activities of the EBV DNA polymerase and cellular DNA polymerase alpha are compared in Table 1. Cellular DNA polymerase alpha was purified from BJAB cells and assayed as described previously (7). Immune serum inhibited the activity of the EBV DNA polymerase but not that of cellular DNA polymerase alpha. The enzyme assays in Table 1 were performed with conditions optimal for the alpha enzyme. When conditions were optimal for the EBV DNA polymerase (100 mM ammonium sulfate), viral enzyme activity was inhibited 90%.

In summary, polyclonal antiserum was elicited in rats and rabbits following immunization with the EBV DNA polymerase isolated from phorbol ester-treated B95-8 cells. Immune serum from both animal species inhibited purified EBV DNA polymerase activity 90%. Cellular DNA polymerase alpha was not inhibited by immune serum to the viral enzyme.

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